UNIVERSITI PUTRA MALAYSIA

THE NUCLEOCAPSID PROTEIN OF NEWCASTLE DISEASE VIRUS AS A CARRIER FOR THE VP1 POLYPEPTIDES OF ENTEROVIRUS 71

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By

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October 2005

Chairman: Professor Datin Khatijah Yusoff, PhD

Faculty: Biotechnology and Biomolecular Sciences

Human enterovirus 71 (EV71) is an important human enterovirus which belongs to the *Enterovirus* genus of the *Picornaviridae* family. Large outbreaks of EV71 have been associated with severe central nervous disease (CNS) manifestations including the hand, foot and mouth disease (HFMD). To date, there is no effective antiviral drug available to treat the infections. Therefore, the development of an effective vaccine is considered as one of the best choice to prevent the diseases caused by EV71.

The VP1 protein which is the most immunogenic capsid protein of EV71, can be used in the development of subunit EV71 vaccines. The complete VP1 protein of EV71 was truncated into six regions and fused to the full length nucleocapsid protein (NPfl) and truncated NP (NPt; which lacks 20% amino acids from its C-terminal end). Western blot analysis using rabbit anti-VP1 serum showed that the N-terminal region of the VP1 protein contained a major antigenic region. Of all

the recombinant NP proteins, the ones carrying truncated VP1 protein, $VP1_{1-100}$ were efficiently expressed in *Escherichia coli* system. Electron microscopic analysis of the purified NPt-VP_{1-100} revealed that this protein predominantly self-assembled into intact ring-like structure whereas NPfl-VP_{1-100} showed disrupted ring-like formations. Rabbits immunized with these purified recombinant proteins exhibited a strong immune response against the complete VP1 protein. The antisera of these recombinant proteins also reacted positively with the authentic EV71 when analyzed by an immunoflourescence assay thus suggesting their potential as subunit vaccine candidates against EV71 infections and also as immunological reagents for the detection of anti-EV71 antibodies in serum samples.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PROTEIN NUKLEOKAPSID VIRUS PENYAKIT SAMPAR AYAM SEBAGAI PEMBAWA POLIPEPTIDA-POLIPEPTIDA VP1 DARIPADA ENTEROVIRUS 71

Oleh

LALITA AMBIGAI SIVASAMUGHAM

Oktober 2005

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Enterovirus 71 (EV71) manusia merupakan sejenis virus manusia yang penting berasal dari genus *Enterovirus* dan famili *Picornaviridae*. Beberapa letusan penyakit yang disebabkan oleh EV71 telah dikaitkan dengan manifestasi penyakit saraf pusat di antaranya ialah penyakit tangan, kaki dan mulut (HFMD). Sehingga kini, tiada ubat yang berkesan untuk merawat jangkitan yang berkaitan. Oleh itu, perkembangan vaksin yang berkesan dianggap sebagai salah satu langkah yang terbaik dalam pencegahan penyakit-penyakit yang disebabkan oleh EV71.

VP1 adalah protein EV71 yang paling immunogenik yang boleh digunakan dalam perkembangan vaksin subunit bagi EV71. Protein VP1 yang lengkap telah dipendekkan kepada enam bahagian dan digabungkan kepada protein nukleokapsid yang lengkap (NPfl) dan protein nukleokapsid protein yang telah dipendekkan (NPt) dari virus penyakit sampar ayam (NDV). Analisis pemblotan

Western dengan menggunakan serum arnab anti-VP1 menunjukkan bahawa bahagian terminal-N protein VP1 mengandungi satu bahagian antigenik yang utama. Daripada kesemua protein-protein rekombinan NP, yang membawa protein VP1, $VP1_{1-100}$ merupakan protein yang diekspreskan dengan paling berkesan dalam sistem *Escherichia coli*. Analisis mikroskopik elektron ke atas NPt-VP_{1-100} menunjukkan bahawa protein in membentuk struktur gegelang yang sempurna manakala NPfl-VP_{1-100} membentuk struktur gegelang yang pecah. Arnab-arnab yang diimunisasikan dengan protein-protein rekombinan ini juga telah menunjukkan tindak balas imun yang kuat terhadap protein VP1 yang lengkap. Antisera protein-protein rekombinan ini juga telah bergerak balas secara positif dengan EV71 yang tulen apabila dianalisis dengan asai imunofloresen lalu mengesyorkan potensi mereka sebagai calon vaksin subunit terhadap penyakit-penyakit EV71 dan juga sebagai reagen imunologik dalam pengesanan antibodi-antibodi anti-EV71 dalam sampel-sampel serum.

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I certify that an Examination Committee met on 10th October 2005 to conduct the final examination of Lalita Ambigai Sivasamugham on her Master of Science thesis entitled "The Nucleocapsid Protein of Newcastle Disease Virus as a Carrier for the VP1 Polypeptides of Enterovirus 71" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis is submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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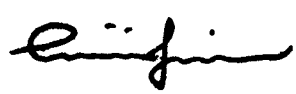

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Date: 8 DEC 2005

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

LALITA AMBIGAI SIVASAMUGHAM

Date: 23/10/05

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AFP	acute flaccid paralysis
bp	base pair
β	beta
BCIP	1-bromo-3-chloro-propane
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
C-terminus	carboxy terminus
dH_2O	distilled water
dNTP	deoxyribonucleic phosphate
DMEM	Dulbecco's Modified Eagle Media
DNA	deoxy-ribonucleic acid
DNase	deoxyribonuclease
DTT	1, 4-dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EV71	Enterovirus 71
F	fusion protein
FCS	fetal calf serum
h	hour
HBV	hepatitis B virus

HBcAg	hepatitis B core antigen
HEPES	n-2-hydroxyethyl-piperazine-n-2-ethanesulfonic acid
HFMD	hand, foot and mouth disease
HN	haemaglutinin-neuraminidase
IgG	immunoglobulin G
IRES	internal ribosome entry site
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
L	large protein
LB	Luria Bertani
µg	microgram
µl	microlitre
µM	micromolar
mA	milliampere
ml	milliliter
mM	millimolar
min	minute
M	molar
NBT	nitro blue tetrazolium
NDV	Newcastle disease virus
ng	nanogram
nm	nanometer

N	normality
NP	nucleocapsid protein
NPfl	full length nucleocapsid protein
NPt	truncated nucleocapsid protein
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
pH	*Puissance hydrogene*
pTrcHis2-NPfl	pTrcHis2 vector carrying the full length NP
pTrcHis2-NPt	pTrcHis2 vector carrying the truncated NP
P	phosphoprotein
PBS	phosphate-buffer saline
PCR	polymerase chain reaction
rpm	revolutions per minute
RBC	red blood cell
RNA	ribonucleic acid
RNase	ribonuclease
s	seconds
SDS	sodium dodecyl sulphate
SPF	specific pathogen free
TAE	tris-acetate-EDTA buffer
TBS	tris-buffered saline
$TCID_{50}$	tissue culture infectious dose

TE	tris-EDTA buffer
U	unit
UTR	untranslated region
UV	ultraviolet
v/v	volume/volume
V	Volt
VLP	virus-like particles
VP1	viral protein 1
VP2	viral protein 2
VP3	viral protein 3
VP4	viral protein 4
VPg	viral protein genomely linked
w/v	weight/volume

CHAPTER 1

INTRODUCTION

Human enterovirus 71 (EV71) belongs to the *Picornaviridae* family and has been noted as one of the causative agents of hand, foot and mouth disease (Chumakov *et al.*, 1979; Nagy *et al.*, 1982; Samuda *et al.*, 1987). The virus enters the host orally via consumption of feacal-contaminated food or water. To date, there is no effective drug treatment or vaccine available for EV71 infections (Shih *et al.*, 2000; Racaniello, 2001; McMinn, 2002). Many clinical and virological similarities between poliovirus and EV71 strongly suggest that the vaccine strategies used against poliovirus can also be adopted to control EV71 infections. As an example, the inactivated poliovirus vaccine (IPV) developed by Jonas Salk was highly effective in reducing the incidence rates of poliomyelitis in 1940s and 1950s (Hull & Aylward, 2001). The same concept was applied to develop an inactivated EV71 vaccine to the Bulgarian epidemic in 1975 but no efficacy data has been obtained (Chumakov *et al.*, 1979). The potential of having virulent revertant virus of live attenuated vaccines (Lin *et al.*, 2002) has made protein-based subunit EV71 vaccine a more favorable choice for vaccine development.

Subunit vaccines are much safer than the inactivated and attenuated vaccines as they cause fewer adverse effects. The isolated subunit protein in a proper conformation can possess neutralizing epitopes. In this regard, two subunit

vaccines were developed based on the immunodominant capsid protein of EV71, VP1 (Wu *et al.*, 2002). The DNA-based vaccine and recombinant protein vaccine were administered to elicit immune response and to protect susceptible newborn mice against lethal EV71 challenge. Both types of vaccines produced similar levels of neutralizing antibodies in the vaccinated dams. With a challenge dose of 230 LD_{50} per mouse, mice born to dams immunized with the recombinant VP1 protein and DNA vaccine showed 80% survival and 40% survival rates respectively. These findings strongly suggest the potential use of VP1 protein of EV71 for the development of effective subunit EV71 vaccines.

Protein carriers have been extensively studied in the development of subunit vaccines. The use of an appropriate carrier can increase the overall yield, the solubility level as well as the immunogenicity of the recombinant proteins (LaVallie & McCoy, 1995). In relation to this, the nucleocapsid protein (NP) of Newcastle disease virus (NDV) has been demonstrated to be a potential molecular carrier of antigenic proteins (Kho *et al.*, 2001; Rabu *et al.*, 2002). The chimeric NP proteins were expressed as highly soluble and stable proteins in the *E. coli* system. In addition, due to the NP protein, the chimeric proteins were also easily purified by sucrose gradient centrifugation and they readily self-assembled into ring-like particles when viewed under an electron microscope. Furthermore, specific pathogen free (SPF) chickens immunized with these chimeric particles elicited an immune response against NDV, suggesting the possible use of NP as

a carrier for immunogens in the development of subunit vaccines and immunological reagents (Rabu *et al.*, 2002).

In view of this, the objectives of the whole study are first, to construct recombinant NP proteins harbouring the polypeptides of VP1 of EV71. Second, to select recombinant NP proteins that are abundantly expressed in soluble form. Third, to evaluate the potential of these recombinant NP proteins in inducing immune response in animals and lastly, to determine the viral neutralization properties of the antibodies raised against these recombinant proteins.

In order to achieve these objectives, the study begun with the cloning of the truncated VP1 fragments encoding several antigenic polypeptides into an expression vector harbouring the NP coding gene. The recombinant proteins were expressed in *E. coli* cells whereby highly expressed and soluble recombinant proteins were subjected to purification by sucrose gradient ultracentrifugation. Electron microscopic analysis was done to observe the structure of these recombinant proteins. The purified recombinant proteins were then used to immunize rabbits and its antigenicity and the immunogenicity were examined by various methods.

CHAPTER 2

LITERATURE REVIEW

2.1 The EV71 Protein: VP1

2.1.1 The History

Human enterovirus 71 (EV71) was first identified in 1969 in California, following its isolation from an infant suffering from encephalitis (Schmidt *et al.*, 1974). The first isolation of EV71 outside of USA was reported in Melbourne, Australia, during an epidemic of aseptic meningitis between 1972 and 1973 (Kennett *et al.*, 1974). In 1975, this virus gained global attention after an outbreak in Bulgaria that resulted in 705 cases of poliomyelitis-like disease and 44 deaths, with most of these cases involving children under the age of five (Chumakov *et al.*, 1979).

The very first association of EV71 with hand, foot and mouth disease (HFMD), was reported only after small epidemics in both Sweden (Blomberg *et al.*, 1974) and Japan (Gobara *et al.*, 1977; Hagiwara *et al.*, 1978) in 1973. Since then, several large epidemics and high-level endemic circulation have been reported in the Asia-Pacific region. The first such epidemic in Malaysia occurred in Sarawak in 1997 (Cardosa *et al.*, 1999), followed by smaller outbreaks in Japan (Komatsu *et al.*, 1999), Peninsular Malaysia (Lum *et al.*, 1998) and Singapore in 1998

(Singh *et al.*, 2000). These outbreaks were associated with numerous cases of HFMD, herpangina, aseptic meningitis, acute flaccid paralysis (AFP), cerebellar ataxia and even the fatal neurogenic pulmonary oedema (Chang *et al.*, 1999) associated with severe brainstem encephalitis (Chang *et al.*, 1998; Huang *et al.*, 1999).

The largest EV71 epidemic outbreak took place in Taiwan in 1998, with more than 100,000 cases of HFMD being reported (Ho *et al.*, 1999). Subsequently, another large outbreak was also reported in Perth, Australia in 1999 (McMinn *et al.*, 2001). Virological studies were done and both EV71 and Coxsackievirus A16 (CA16) were isloated from these outbreaks but the former was the predominant virus. EV71 continues to circulate in the Asia-Pacific region and in February 2003, the virus was again reported in Sarawak, Malaysia. Most of the cases reported were uncomplicated HFMD but a small number did have neurologic disease (Cardosa *et al.*, 2003).

2.1.2 Virus Classification

Enterovirus 71 belongs to the family *Picornaviridae*, genus *Enterovirus* and the species *Human enterovirus A*. Initially, the original 64 human enterovirus serotypes were grouped into polioviruses (PV), coxsackieviruses A (CA), coxsackieviruses B (CB) and echoviruses (EV) 68 to 71. The major characteristic